

**METHOD ~~AND APPARATUS~~ FOR RAPIDLY ASSAYING
ALDEHYDE-CONTAINING DISINFECTANT**

5

Background of the Invention

Field of the Invention

The field of the invention relates to a method and device to detect a point of interest of an aldehyde in a test sample such as a disinfectant.

Description of the Related Art

10 General methods to determine *o*-phthalaldehyde (OPA) or glutaraldehyde concentrations are mainly instrumental measurements that could be classified into chromatographic measurement (chromatographic, HPLC analysis) or non-chromatographic measurement (direct spectroscopic assay). For HPLC analysis, OPA or glutaraldehyde are measured by both a derivative method or a non-derivative method.

15 The most common derivative method is to convert OPA or glutaraldehyde to 2,4-dinitrophenylhydrazones by reacting OPA with 2,4-dinitrophenylhydrazine. Since the UV absorption is greatly enhanced, this method is valuable for low level OPA or glutaraldehyde measurements especially in environmental analysis. For measurements of high concentrations of OPA or glutaraldehyde, such as the OPA or glutaraldehyde disinfectants, OPA or glutaraldehyde could be measured directly without making derivatives first. OPA or glutaraldehyde may be analyzed easily with GC analysis. For non-chromatographic analysis, OPA or glutaraldehyde could be measured directly with spectrophotometric methods. However, one drawback to this method is that there must be no interference at the specific wavelength used. For example, OPA or glutaraldehyde

20 could be oxidized slowly by air and the carboxylic acid formed may interfere in such assays.

25

30 All three instrumental methods involve the preparation of samples and use of an instrument. They are all time-consuming and too expensive or too complicated for hospital end users. Therefore, Albert Browne and 3M have developed a simple strip procedure for a Pass/Fail test. In such a test, the strip was dipped into either OPA or glutaraldehyde solutions for a certain amount of time. After a predetermined time, the

strip color was compared with some standard colors. Their strip chemistry principles were not released. The problems with this method are consistency and accuracy. The strip method has the following problems (1). Good solutions (OPA or glutaraldehyde higher than "POI", the point of interest) often fail the test for different reasons. (2). The soaking time and waiting time have to be controlled carefully. Any deviation will lead to different shades of color and a false reading. (3). Moving of the strip when soaking will lead to the loss of chemical reagents to the OPA or glutaraldehyde solutions leading to false reading. (4). Individual users have different color recognition habits and often have a different opinion of the end-color. (5). The final color is dependent on many factors and is particularly sensitive to time.

The current invention provides another method without the above problems. Although the chemistry principle could also be used for the strip approach, in a preferred embodiment it is used for the color change of a solution.

Summary of the Invention

The present invention is drawn to a method of determining the presence of a point of interest of an aldehyde in a test sample which includes the steps of:

- 1) reacting the aldehyde in the test sample with an amount of a compound that reacts with a carbonyl group of the aldehyde in a first reacting step, wherein said amount is sufficient to react with the aldehyde to the point of interest to produce a first color;
- 2) reacting a compound having an amino group with any remaining aldehyde in the test sample in a second reacting step, the compound being one that reacts with the aldehyde to produce a second color; and
- 3) determining the presence of an excess of aldehyde in the test sample to the point of interest by observation of a final color of the test sample.

In a preferred embodiment, the compound having an amino group is an amino acid. In a more preferred embodiment the amino acid is glycine or lysine. In a preferred embodiment, the compound that reacts with the carbonyl group of the aldehyde is selected from the group including a salt of bisulfite, a salt of cyanide, hydrazine, and hydroxylamine.

In a preferred embodiment, the aldehyde includes a germicide. In a more preferred embodiment, the germicide is selected from the group including OPA, glutaraldehyde, and formaldehyde.

In one embodiment, the compound having an amino group is mixed with the test sample at the same time as the compound that reacts with the carbonyl group of the aldehyde. In an alternate embodiment, the compound having an amino group is added to the test sample after the compound that reacts with the carbonyl group of the aldehyde.

The first color produced by the first reacting step may be colorless.

In one embodiment, less than 1% of the aldehyde remains after the first reacting step when the amount of aldehyde in the test sample is less than the point of interest. The method may also include drawing up a fixed volume of an aldehyde-containing test sample before or during the first reacting step. Furthermore, the fixed volume may be loaded to a measuring device having a gas or vapor permeable but liquid impermeable membrane. In a preferred embodiment, the fixed volume may be loaded to a measuring device containing the compound for the first reacting step or the compound for the second reacting step.

Another aspect of the invention pertains to a liquid measuring device including at least one compartment for determining the presence of a point of interest of an aldehyde in a test sample including a first compartment having a proximal and distal end which contains an amount of a first compound that reacts with a carbonyl group of the aldehyde in a first reacting step, wherein the amount is sufficient to react with the aldehyde to the point of interest to produce a first color. In a preferred embodiment, the first compartment further includes a compound having an amino group that reacts with the aldehyde to produce a second color. The liquid measuring device may optionally include a second compartment in liquid communication with said first compartment by means of a valve. In one embodiment, the valve is a one-way valve. In an alternate embodiment, the valve is an on/off valve. In a preferred embodiment, the second compartment of the liquid measuring device includes a compound having an amino group that reacts with the aldehyde to produce a second color.

In a preferred embodiment, the liquid measuring device may be either a syringe or pipet. In a preferred embodiment, the liquid measuring device includes a gas or vapor permeable but liquid impermeable membrane between the proximal and distal end of the first compartment.

5 In one embodiment, the liquid measuring device includes a filter at or near the distal end of the first compartment. Additionally, the liquid measuring device may also include a valve at or near the distal end of the first compartment. In one embodiment, the valve is a one-way valve. In an alternate embodiment, the valve is an on/off valve.

10 Optionally, the liquid measuring device may include a needle assembly. In a preferred embodiment, the needle assembly includes a needle cap.

In a most preferred embodiment, the aldehyde in the test sample is selected from the group including OPA, glutaraldehyde, and formaldehyde.

15 For purposes of summarizing the invention and the advantages achieved over the prior art, certain objects and advantages of the invention have been described above. Of course, it is to be understood that not necessarily all such objects or advantages may be achieved in accordance with any particular embodiment of the invention. Thus, for example, those skilled in the art will recognize that the invention may be embodied or carried out in a manner that achieves or optimizes one advantage or group of advantages as taught herein without necessarily achieving other objects or advantages as may be taught or suggested herein.

20 Further aspects, features and advantages of this invention will become apparent from the detailed description of the preferred embodiments which follow.

Brief Description of the Drawings

25 These and other feature of this invention will now be described with reference to the drawings of preferred embodiments which are intended to illustrate and not to limit the invention.

Figure 1 shows the basic principles of the described assay. Reaction 1 shows the reaction of aldehyde with compound X to produce a compound with a first color. Preferably, the first color is colorless. Reaction 2 shows the reaction of aldehyde and Y to form a compound with a second color. Preferably, reaction 2 is slower than Reaction

30

1. If the concentration of aldehyde is below the POI (point of interest) only compound X will react and the resulting solution will be the first color as shown in the bottom half of the figure. In the presence of a level of aldehyde that is equal to or more than the POI, a solution with the second color or the combined color of the first color and the second color will be formed.

Figure 2 shows a pipette and two variants of a syringe with a gas or vapor permeable liquid impermeable barrier.

Figure 3A shows the coupling of the gas or vapor permeable liquid impermeable barrier to the syringe or pipette. Figure 3B illustrates how inserts 4 at the top of the pipette or syringe attach the gas or vapor permeable liquid impermeable barrier to the pipette or syringe. Figure 3C illustrates a holder 5 that holds the inserts in place. Figure 3D shows the inserts and the coupling of the gas or vapor permeable liquid impermeable barrier.

Figure 4 is an expanded view of figure 3C which shows a gas or vapor permeable liquid impermeable barrier 1, an insert 4, and a holder 5.

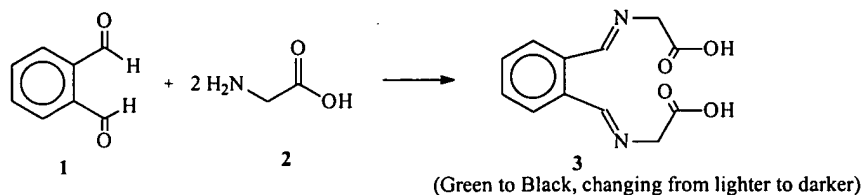
Figure 5 shows one embodiment of the invention where the position of the gas or vapor permeable liquid impermeable membrane is adjusted by means of a screw.

Figures 6A and 6B show embodiments of the liquid delivery apparatus with all chemicals in one chamber. Figure 6C shows a two chambered embodiment of the liquid delivery apparatus. The test sample may be taken into the first chamber for reaction with the first compound such as compound X in Figure 1. Then the sample is moved by means of a one-way valve or a manual ON/OFF valve 8 into the second chamber where the test sample reacts with the second compound such as compound Y of Figure 1.

Detailed Description of the Preferred Embodiment

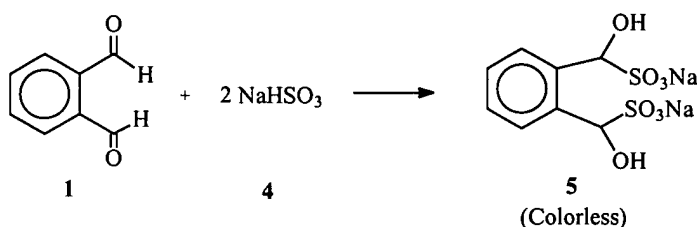
While the described embodiment represents the preferred embodiment of the present invention, it is to be understood that modifications will occur to those skilled in the art without departing from the spirit of the invention. The scope of the invention is therefore to be determined solely by the appended claims.

Aldehydes react with amino-containing compounds like amino acids or amines to form an imine or more commonly known as a Schiff's base, which is often colored. Taking glycine as an example:



Schiff's Base Formation between OPA and Glycine

Another known aldehyde reaction is the sodium bisulfite carbonyl addition reaction.



Addition Reaction of Sodium Bisulfite to OPA

The sodium bisulfite addition reaction is more favorable than that of Schiff's formation since the former reaction is fast and hard to reverse. Thus, in the presence of both a compound containing an amino group such as an amino acid and a reagent such as sodium bisulfite, the aldehyde will react first with sodium bisulfite and then with the amino acid. Therefore, it is possible to design a color pass/fail reaction by controlling the amount of reagents to react with aldehydes such as formaldehyde, OPA or glutaraldehyde. The key is the amount of reagent such as sodium bisulfite which is designed to react with the aldehyde without a color being developed in the presence of an amino acid. Any remaining aldehyde will then react with the amino acid to develop a colored solution. This confirms the presence of a certain amount of an aldehyde such as formaldehyde, OPA or glutaraldehyde in a test solution such as a disinfectant

solution. On the other hand, if no color was developed, it confirms that the formaldehyde, OPA or glutaraldehyde concentration is below an acceptable specification. The specific concentration can be set to any point by adjusting the amounts of the chemical reagents used or by using different amounts of aldehyde (formaldehyde, OPA or glutaraldehyde) in the test solution.

Thus, a color pass/fail reaction for determination of excess aldehyde by control of reagents which react with aldehyde is described. The key is the amount of reagent such as sodium bisulfite which is designed to react with the Point of Interest (POI) level of aldehyde without a color being developed in the presence of a compound containing an amino group such as an amino acid. Any "extra" aldehyde, exceeding the POI, will then react with the compound containing an amino group, causing a color to be developed. In a preferred embodiment, the aldehyde is either OPA or glutaraldehyde and the compound containing the amino group is an amino acid. This method is especially useful for quality control where components only needed to be examined in pre-determined ranges.

A number of reagents which are known to react quickly with aldehydes may be used in the practice of the invention. These include any chemicals which can oxidize or reduce the aldehyde group and any chemicals which can react with and alter the carbonyl functional group of the aldehyde. Examples of such reagents are disclosed in Morrison & Boyd, "Organic Chemistry", Chapter 19, Allyn and Bacon, 3rd edition, 1973, which is herein incorporated by reference. Such reagents include, but are not limited to, $\text{Ag}(\text{NH}_3)_2$; KMnO_4 ; $\text{K}_2\text{Cr}_2\text{O}_7$; H_2 + Ni, Pt, or Pd; LiAlH_4 or NaBH_4 , then H^+ ; Zn (Hg), conc. HCl; NH_2NH_2 , base; Grignard reagents; salts of cyanide and bisulfite; ammonia derivatives such as hydroxylamine, hydrazine, phenylhydrazine, and semicarbazide; reactions with alcohols in the presence of acid; and reactions with acid or base such as the Cannizzaro reaction, the aldol condensation, and the Perkin condensation. In a preferred embodiment, the reagent which reacts with the aldehyde is a salt of either bisulfite or cyanide.

This aspect of the invention is illustrated in Figure 1. Both compounds X and Y react with the aldehyde in the figure. Preferably X reacts much faster than Y. Preferably, the reaction of X with aldehyde results in a colorless compound whereas the

reaction of Y with aldehyde results in a colored compound. A point of interest is chosen and the amount of X that will react with the point of interest is determined. When the aldehyde is mixed with X and Y, the aldehyde will react first with compound X which is kinetically and thermodynamically favored. Any excess aldehyde will then react with compound Y to form a colored solution. Consequently, if a colored solution results, the concentration of aldehyde is above the point of interest. The determination may be made visually, with or without a color chart. Alternatively, a spectrophotometer may be used. If the reaction between the aldehyde and compound X is not kinetically and thermodynamically favored, then compound Y can be added after the aldehyde reacts with compound X as shown in Figure 1.

The theoretical amount of OPA: sodium bisulfite is 1:2. However, it was found that less sodium bisulfite is needed to react with OPA than the theoretical amount in order to get a good color display.

Another aspect of the invention is a liquid-measuring device, such as a pipette or syringe, for carrying out the assay. This device could be used for any "fixed-volume" measurement and transfer in chemistry, biochemistry, clinical chemistry or other industries.

The apparatus may be a syringe or pipette with one or more barrels and plungers and a membrane barrier with or without a coupling device. The membrane barrier is a gas or vapor permeable and liquid impermeable barrier. In the presence of certain pressure differences between the two sides of the barrier, the gas or vapor flows through the membrane but not the liquid. Any suitable gas or vapor permeable and liquid impermeable materials can be used for this purpose. Some examples include, but are not limited to, nonwoven polyolefin, such as Tyvek™ (non-woven polyethylene), or CSR (non-woven polypropylene central supply room), wrapping material and any other hydrophobic filtering materials. Optionally, the device contains an insert and a holder. The syringe or pipette apparatus may also contain valves to control the flow of liquid.

The membrane barrier can be thermally bound to the syringe or pipet. It can also be attached to the syringe or pipet with an adhesive or connected to the syringe barrel by a coupling device. The coupling device may be connected to an insert for

altering the position of the membrane barrier. The position of the membrane barrier can be adjusted by the length of the insert. The insert may be secured with a holder.

The membrane barrier is a gas or vapor permeable but liquid impermeable barrier. The membrane barrier is positioned such that the liquid can only be filled up to the barrier. The invention has several preferred embodiments.

In the first embodiment (Figure 2), a gas or vapor permeable liquid impermeable membrane 1 is fixed into the pipette 7 or syringe 6 and held in place at the desired maximum volume by means known in the art. The syringe includes a plunger 3. The syringe can have a metal or plastic needle with or without a needle cap. In one embodiment (Figures 3A-3D), a coupling device 2 is used which is larger or smaller than the diameter of the pipette 7 or syringe 6. Two parts of the pipet or syringe with different lengths can be joined together with such a coupling device.

Coupling of the membrane barrier to the syringe or pipette is shown in Figures 3A, 3B, 3C, 3D and Figure 4. The membrane can be inserted into the syringe or pipet from the top of the pipette or syringe by an insert 4 which may be secured with a holder 5 and its position varied by any means known in the art such as by a screw (Figure 5) or a slidable adjustment (Figure 4). Figure 3D shows an insert which has a larger diameter than the pipette or syringe. By adjusting the insert and creating a negative pressure on the upper part of the pipette or syringe, the fluid can be loaded into the syringe or pipette up to the barrier.

Figures 6A, 6B and 6C illustrate the use of the measuring device with this invention. Figures 6A and 6B show a syringe with a gas or vapor permeable liquid impermeable barrier and two chemicals. The liquid can be filled in the syringe by inserting the plastic needle into the sample solution, pulling the plunger to create a negative pressure in the syringe, and loading the liquid into the syringe. The measuring device can have a filtering material (Figure 6A) or valve (Figure 6B) to retain the chemicals in the barrel. The chemical in the syringe can be in either a liquid or solid form. The valve can be a one-way valve or a manual ON/OFF valve.

Figure 6C provides another embodiment for mixing more than one reactant successively. It has two chambers 9, 10. A fixed volume of any solution including, but not limited to an aldehyde is drawn up through a one-way valve or an ON/OFF valve 8

into the first chamber 9 where it mixes with the first reactant, for example sodium bisulfite. After a predetermined time, the reactants flow through a second one-way valve or an ON/OFF valve 8 into a second reaction chamber 10 which might contain an amine such as lysine, for example, to complete the reaction. Alternatively, a three-way valve can be used instead of two one-way valves.

The invention has several advantages over the prior art methods. First, the pass/fail conclusion is consistent and convenient. Preferably, there is no need to guess the color. The user's only conclusion will be "colored" or "not colored." Second, the liquid transferring device is consistent and convenient. A fixed volume of liquid can be taken by a simple operation. Third, the solution color is easier to visualize than a test strip paper since the test strip paper itself is colored, leading to false positive results. Fourth, the color displaying time can be adjusted by adding a base to make the reaction faster or an acid to make the reaction slower. Fifth, the color being displayed can be adjusted by choosing different amino acids or amines. Sixth, the darkness of the color being displayed can be adjusted by the amount of the amino acids or amines. Seventh, the assay is extremely easy to run and interpret. And finally, the liquid transferring device could be used for any "fixed-volume" transfer in chemistry, biochemistry, clinical chemistry or other industries.

EXAMPLES

Example 1. Effect of OPA to Sodium Bisulfite mole ratio (0.5:1 to 8:1)

Sodium bisulfite, glycine and OPA were added in sequence. The OPA to sodium bisulfite mole ratio was adjusted from 0.5:1 to 8:1 (Table 1). Table 1 shows that the solution with a 2:1 ratio developed a color while a 1:1 ratio did not show color in one week.

It was found that less than the theoretical amount of sodium bisulfite was needed to react with the OPA. This indicates the OPA solutions in this concentration region can be differentiated by observing the color of the solution after a specified time (as in Vial 2 and Vial 3). Since we can control the volume of OPA in testing, we can theoretically test an OPA solution in any concentration range.

Table 1.

	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5
NaHSO ₃ (82mM)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)
Glycine (82mM)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)
OPA (0.55%, 41mM)	200μL (0.00820 mMole)	400μL (0.0164 mMole)	800μL (0.0328 mMole)	1600μL (0.0656 mMole)	3200μL (0.1312 mMole)
OPA:NaHSO ₃ mole ratio	0.5:1	1:1	2:1	4:1	8:1
Time to develop color	>1week	>1week	4' 45''	65''	35''
Initial color	Colorless	Colorless	Light yel/grn	yellow/grn	yel/grn
Final color (after 30')	Colorless	Colorless	Dark green	Between	Dark Blck

Example 2. Effect of OPA to Sodium Bisulfite mole ratio (1:1 to 2:1).

Sodium bisulfite, glycine and OPA were added and the OPA to sodium bisulfite mole ratio was adjusted as in Example 1. Table 2 shows three points of interest (POI). The first POI, was the 2:1 mole ratio, the second POI was the 1.75:1 mole ratio and the third POI was the 1.5:1 mole ratio of OPA to sodium bisulfite. For the 2:1 ratio, 5 minutes were needed to display the initial color. For the 1.75:1 ratio, 13 minutes were needed to display the initial color. For the 1.5:1 ratio, color was not displayed for a few days.

Table 2.

	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5
NaHSO ₃ (82mM)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)
Glycine (82mM)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)
OPA (0.55%, 41mM)	400μL (0.00164 mMole)	500μL (0.0205 mMole)	600μL (0.0246 mMole)	700μL (0.0278 mMole)	800μL (0.0328 mMole)
OPA:NaHSO ₃ mole ratio	1:1	1.25:1	1.5:1	1.75:1	2:1
Time to develop color	Never	Never	Never	13'	5'
Initial color	Colorless	Colorless	Colorless	Very light Pink	(Light) Yel/Grn
Final color (after 30')	Colorless	Colorless	Colorless	Green	Dark Grn

In Table 2, the reaction volume is varied by varying the amount of OPA solution from 400 μl to 800 μl. The assay is independent of volume. The OPA to sodium bisulfite mole ratio is a key parameter of the assay.

Example 3. OPA Concentration Variation Study in the OPA to Sodium Bisulfite Mole Ratio 1:1 to 2:1 Region. (same volume different concentration)

Sodium bisulfite, glycine and OPA were added and the OPA to sodium bisulfite mole ratio was adjusted as in Example 2. As shown in Table 3, the first POI was in the range of 6'20''-7'20'' range and the time needed for color change was very consistent. However, for the second POI, there was some variation for this time (17-24'). Without being bound by any mechanism, this may be due to the visual limitation or it may mean that at diluted concentration, the color development is more likely to be influenced by micro reaction condition variations, such as temperature, pH or even the exposure of sunlight.

Table 3.

	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5
NaHSO ₃ (82mM)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)
Glycine (82mM)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)
OPA (%)	0.275 (20.50 mM)	0.344 (25.63 mM)	0.413 (30.75 mM)	0.481 (35.88 mM)	0.550 (41.00 mM)
ml (0.55%OPA) to dilute to 100ml with water	50.00	62.50	75.00	87.50	No dilution
OPA solution used	800μl	800μl	800μl	800μl	800μl
OPA mMole	0.0164	0.0205	0.0246	0.0287	0.0328
OPA:NaHSO ₃ mole ratio	1:1	1.25:1	1.5:1	1.75:1	2:1
Time to develop color	Never	Never	Never	17-- 20'	6' 20" --7' 20"
Time to develop color, repeat #1	Never	Never	Never	18-- 21'	6' 20" --7' 20"
Time to develop color, repeat #2	Never	Never	Never	19-- 21'	6' 20" --7' 20"
Time to develop color, repeat #3	Never	Never	Never	21-- 23'	6' 20" --7' 20"
Time to develop color, repeat #4	Never	Never	Never	22-- 24'	6' 20" --7' 20"
Initial color	Colorless	Colorless	Colorless	Very light pink	(Light) Yel/Grn
Final color (after 2h)	Colorless	Colorless	Colorless	Dark Grn	Dark Grn

[illegible]

5

Table 4.

	Vial 1	Vial 2	Vial 3
NaHSO ₃ (82mM)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)
Glycine (82mM)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)
OPA (% , 41mM)	0.275	0.344	0.413
ml (0.55%OPA) to dilute to 100ml	50.00	62.50	75.00
OPA solution used	1201μl (0.0246 mMole)	1119μl (0.0287 mMole)	1065μl (0.0328 mMole)
OPA:NaHSO ₃ mole ratio	1.5:1	1.75:1	2:1
Time to develop color (up to 30')	Never	16'	5'
Initial color	Colorless	(light) Yel/Grn	(Light) Yel/Grn

Thus, one of the key factors for this invention is the mole ratio of aldehyde to sodium bisulfite. Similar results were obtained for DL-alanine, ε-amino-n-caproic acid and L-lysine, except that different end colors were observed.

Example 5: Further experiments with OPA for POI's in the range of 0.35% and 0.30%.

Changes due to the type of amino acid and the mole ratio were illustrated in the following example where DL-dopa is used as the amino acid (also see Example 7). Sodium bisulfite, and OPA were added as in Example 1. DL-dopa was substituted for glycine as the amino acid.

Table 5.

82mM NaHSO ₃	Saturated DL-dopa	0.35% OPA (23.09mM)	0.30% OPA (22.37mM)	0.35% OPA (23.09mM) Color (minutes and seconds) in	0.30% OPA (22.37mM) Color (minutes and seconds) in
100μl (0.0082mMole)	100μl	450μl (0.0119 mMole)	450μl (0.0101 mMole)	2'20"-2'30"	3'20"-4'
100μl (0.0082mMole)	100μl	400μl (0.0106 mMole)	400μl (0.0089 mMole)	3'00"-3'30"	5'-10'
100μl (0.0082mMole)	100μl	390μl (0.0103 mMole)	390μl (0.0087 mMole)	3'40"-4'10"	5'30"-11'

In the above example, the use of DL-dopa as the amine resulted in an orange color. The type of amino acid, mole ratio, and reaction time are all important to determine the formation of color.

Example 6. Base Effect for the Color Development Time.

This example shows that added base promotes the reaction rate so that the color displaying time can be shortened. Thus, a certain amount of base could be added to display the color within a desired period of time.

Table 6.

	Vial 1	Vial 2	Vial 3	Vial 4
NaHSO ₃ (82mM)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)
Glycine (82mM)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)
OPA (%) (variation conc.)	0.275	0.344	0.413	0.481
ml (0.55%OPA), added to dilute to 100ml	50.00	62.50	75.00	87.50
OPA (0.55%, 41mM)(initial conc.)	800μl (0.0164 mMole)	800μl (0.0205 mMole)	800μl (0.0246 mMole)	800μl (0.0287 mMole)
OPA:NaHSO ₃ mole ratio	1:1	1.25:1	1.5:1	1.75:1
Time to develop color (without NaOH)	colorless	colorless	colorless	Very light pink (17'-24')
Time to develop color (100μL NaOH added)	1.5h slight yellow	1h slight yellow	2' yellow	<2', yellow
Time to develop color (200μL NaOH added)	All turned yellow in less than 1'. Too fast. Too much base.			

Note: Sodium hydroxide was added before OPA.

Conversely, it was found that added acid, such as citric acid, would delay the color display. This would be useful in the case if the color is displayed too soon (data not shown).

Example 7. Other Amino Acids with Added Base (100μL).

It was found with other amino acids that the displayed colors were different. For example, when reacting with OPA, DL-alanine was bright yellow and for ε-amino-*n*-caproic acid, the color was pink. Furthermore, the reaction rates were also different. Thus both DL-alanine and ε-amino-*n*-caproic acid displayed color significantly later than glycine (data not shown).

Example 8. Activated Cidex solution (containing 2.1% glutaraldehyde) with Lysine

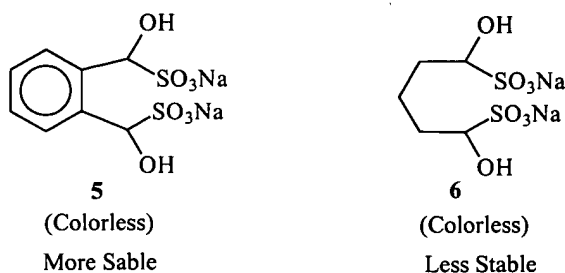
To five scintillation vials, glutaraldehyde, sodium bisulfite and lysine were added and mixed. A yellow color developed gradually from Vial 5. No color was observed in Vial 1. The “between” colors were seen from Vial 2 to Vial 4 but they are so “gradual” that they could not be distinguished visually.

5 **Table 7.**

	Vial 1	Vial 2	Vial3	Vial 4	Vial 5
NaHSO ₃ (82 mM)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)
Lysine (82mM)	1600 μl (0.1312 mMole)	1600 μl (0.1312 mMole)	1600 μl (0.1312 mMole)	1600 μl (0.1312 mMole)	1600 μl (0.1312 mMole)
Glutaraldehyde (220mM) solution used	74.5 μl (0.0614 mMole)	93.2 μl (0.0205 mMole)	111.8 μl (0.0246 mMole)	130.5 μl (0.0287 mMole)	149.1 μl (0.0328 mMole)
Glutaraldehyde:NaHSO ₃ mole ratio	1:1	1.25:1	1.5:1	1.75:1	2:1
Color at 15 minutes	Colorless	Very light yellow to yellow, very gradual. No clear-cut difference			Yellow

5

This can be explained in light of the stabilities of the compounds involved. First, if aldehyde-sodium bisulfite complex **5** is more stable than aldehyde-sodium bisulfite complex **6**, we would see a larger POI range from glutaraldehyde.

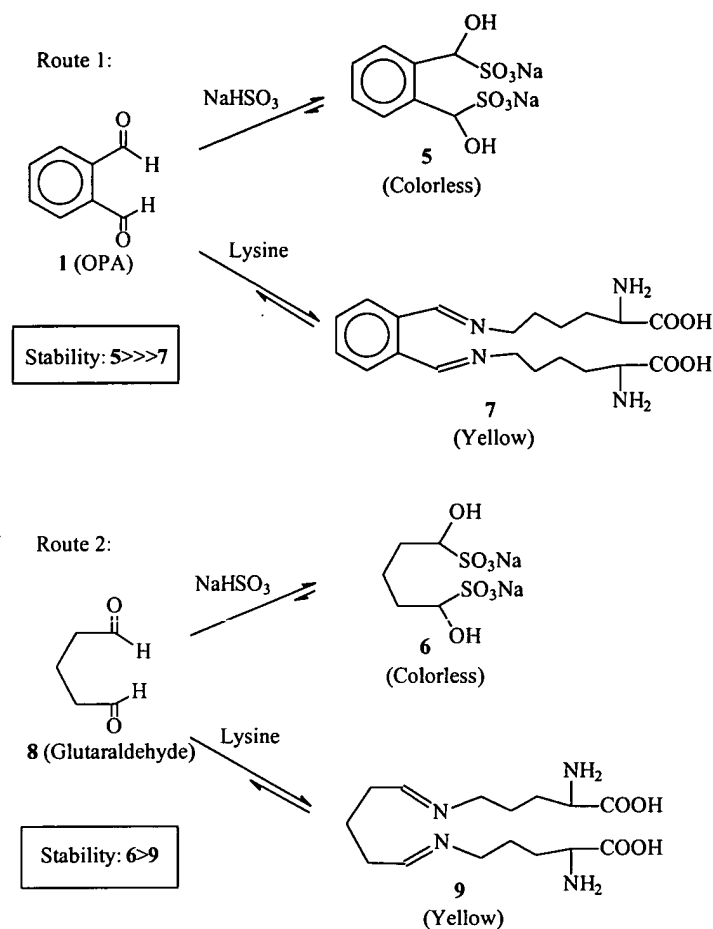


10

The Ranges of POI Are Related to the Stability of Compound **5** and **6**.

15

Or in more accurate terms, the different POI ranges from OPA and glutaraldehyde might be a result of the competence of aldehyde-sodium bisulfite formation and the aldehyde/amino acid Schiff's base formation both kinetically and thermodynamically.



5

In Route 1, when the three components are mixed together, the formation of compound **5** is more favorable than the formation of compound **7**, both kinetically and thermodynamically.

This is somewhat different in the situation of Route 2. Although the formation of **6** is still more favorable than that of **9**, the difference is much smaller than that between **7** and **5** in Route 1. Therefore if the three components (glutaraldehyde, sodium bisulfite and lysine) are mixed, depending on the ratio, there may be some small amount of **9** formed which results in a detectable yellow color. However, this situation is manipulated by mixing of compound **8** and NaHSO₃ first and adding lysine last. In this case, if there is no aldehyde left, lysine must compete with **6** to form **9**, which is not very favorable. With some combinations of amino acid and aldehyde, the order of

adding the reactants may be important. In the following example, the amino acid was added last.

Example 9. Amino acid was added last

5

To five scintillation vials, glutaraldehyde and sodium bisulfite were added and mixed first, and lysine solution was added last respectively. A yellow color developed gradually from Vial 5 to Vial 2 but not in Vial 1 (Table 8).

Table 8. Color development in five scintillation vials. The color developed gradually from Vial 5 to Vial 2 but not in Vial 1.

Table 8.

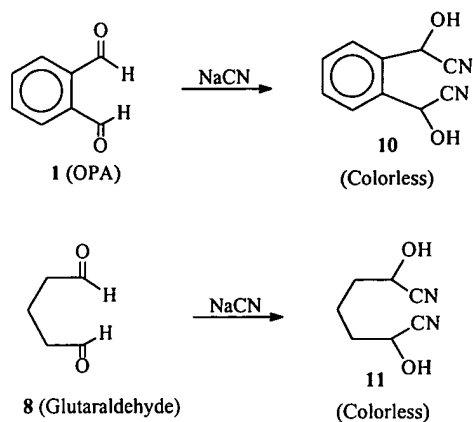
	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5
NaHSO ₃ (82mM)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)
Lysine (82mM)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)
Glutaraldehyde (220mM) solution used	74.5μl (0.0614 mMole)	93.2μl (0.0205 mMole)	111.8μl (0.0246 mMole)	130.5μl (0.0287 mMole)	149.1μl (0.0328 mMole)
Glutaraldehyde: NaHSO ₃ mole ratio	1:1	1.25:1	1.5:1	1.75:1	2:1
Color at 15'	Colorless	light yellow	yellow	yellow	yellow

A narrower POI range was observed for glutaraldehyde reacting with lysine and sodium bicarbonate. Adding the amino acid (lysine) last was the key. Table 8 shows a clear color difference between Vial 1 (color less) and Vial 3 (yellow). Thus by allowing the glutaraldehyde and sodium bisulfite to react first and then adding lysine, results are similar to those observed with OPA above.

Depending on the chemicals used, the time may vary. For NaHSO₃, the lysine can be added immediately after the aldehyde is mixed with the NaHSO₃. Thus the assay described can be applied generally to aldehydes and amines to provide a pass/fail type assay of aldehyde content.

Example 10.

The above chemistry principle may be applied in the reaction of aldehydes and compounds containing an amino group generally. This example shows the reaction of glutaraldehyde and sodium cyanide using either glycine or lysine as the amino acid. The formation of corresponding two aldehyde cyanide addition compounds are shown as below.



The Formation of Colorless Aldehyde-Cyanide Addition Compounds 10 and 11

- 5 To each of the 5 scintillation vials, glutaraldehyde and sodium cyanide were added and mixed first (Table 9), and lysine solution was added last. A yellow color developed from Vial 5 but not from the other vials. A POI was identified between Vial 4 and Vial 5.

Table 9.

5 Glutaraldehyde : Sodium Cyanide Mole Ratio (0.125:1 to 2:1).

	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5
NaCN (82mM)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)
Glycine (82mM)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)
Glutaraldehyde (220mM) solution used	9.3μl (0.0020 mMole)	18.6μl (0.0041 mMole)	37.3μl (0.0082 mMole)	74.5μl (0.0164 mMole)	149.1μl (0.0328 mMole)
Glutaraldehyde:NaCN mole ratio	0.125:1	0.25:1	0.5:1	1:1	2:1
Final color in 7'	Colorless	Colorless	Colorless	Colorless	Yellow

Example 11

10 To each of the 5 scintillation vials, glutaraldehyde and sodium cyanide were added and mixed first, and lysine solution was added last (Table 10). A yellow color developed from Vial 2 to Vial 5 but not recognizable from Vial 1. A POI was identified between Vial 1 and Vial 3. It is only practical with the naked eye to differentiate the colors between Vial 1 and Vial 3. That is, it would be challenging to distinguish the
15 difference between Vial 1 and Vial 2 or between Vial 2 and Vial 3. Thus we may conclude that no narrower POI could be identified unless an instrument is employed.

Table 10.

5 Glutaraldehyde : Sodium Cyanide Mole Ratio (1:1 to 2:1).

	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5
NaCN (82mM)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)	200μl (0.0164 mMole)
Glycine (82mM)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)	1600μL (0.1312 mMole)
Glutaraldehyde (220mM) solution used	74.5μl (0.0164 mMole)	93.2μl (0.0205 mMole)	111.8μl (0.0082 mMole)	130.5μl (0.0164 mMole)	149.1μl (0.0328 mMole)
Glutaraldehyde:NaCN mole ratio	1:1	1.25:1	1.5:1	1.75:1	2:1
Time to develop color	Never	3'	2'	1'	1'
Color in ~8 minutes	Colorless	Very light Yellow	Yellow	Yellow	Yellow

10 The aldehyde solution can be measured and transferred by means known in the art such as by a regular pipet or syringe. In a preferred embodiment, the aldehyde solution can be measured and transferred using a liquid measuring device as described herein which features a gas or vapor permeable, liquid impermeable, membrane. The use of the liquid measuring device containing the gas or vapor permeable, liquid impermeable membrane of the present disclosure has the advantage that the liquid can be transferred easily using a simple operation with consistent results.

15 Compound X and Compound Y (Figure 1) may be in one vial or in two separate vials. They may be transferred using either a pipet or syringe. The aldehyde may be added to compound X and the resulting mixture added to compound Y, the aldehyde may be added to compounds X and Y together, or the aldehyde and chemical Y can be added to the chemical X consecutively. The measuring and/or transferring of the aldehyde test sample can be conducted with a regular pipet or syringe. The gas or vapor permeable liquid impermeable barrier adds many benefits as described previously.

20 In one embodiment, shown in Figure 6C, the Compound X may be in a first chamber 9. The aldehyde is drawn up through the valve 8, up to the gas or vapor

permeable liquid impermeable barrier 1. After a predetermined time, the aldehyde and compound X are transferred to a second chamber 10, through a valve 8 which is either a one-way or an on/off valve, where they react with compound Y. After a pre-determined time, the color in the second chamber 10 is observed and the presence or absence of excess aldehyde in the test sample determined.

It will be understood by those of skill in the art that numerous and various modifications can be made without departing from the spirit of the present invention. Therefore, it should be clearly understood that the forms of the present invention are illustrative only and are not intended to limit the scope of the present invention.

10

11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2184
2185
2186
2187
2188
2189
2190
2191
2192
2193
2194
2195
2196
2197
2198
2199
2200
2201
2202
2203
2204
2205
2206
2207
2208
2